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Oxidative stress and macrophages: driving forces behind exacerbations of asthma and COPD?

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Running head: Oxidative stress and macrophages in asthma and COPD

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Abstract

Oxidative stress is a common feature of obstructive airway diseases like asthma and chronic obstructive pulmonary disease (COPD). Lung macrophages are key innate immune cells that can generate oxidants and are known to display aberrant polarization patterns and defective phagocytic responses in these diseases. Whether these characteristics are linked in one way or another and whether they contribute to the onset and severity of exacerbations in asthma and COPD remains poorly understood. Insight into oxidative stress, macrophages and their interactions may be important in fully understanding acute worsening of lung disease. This review therefore highlights the current state of the art regarding the role of oxidative stress and macrophages in exacerbations of asthma and COPD. It shows that oxidative stress can attenuate macrophage function, which may result in impaired responses towards exacerbating triggers and may contribute to exaggerated inflammation in the airways.

Introduction

Obstructive lung diseases such as asthma and chronic obstructive pulmonary disease (COPD) are characterized by chronic lung inflammation of diverse origin and localization, but both are associated with oxidative stress and changes in macrophage function (113, 128, 129, 155, 157). Macrophages are the most abundant leukocytes in the airways and crucial for regulating immune responses. In addition, they are well known for their ability to generate reactive oxidants, like reactive oxygen species (ROS) and reactive nitrogen species (RNS), to protect against invading pathogens (69). The host protects itself against these reactive species by increased expression of antioxidants. Oxidative stress results from an imbalance between the production of oxidants and antioxidant defenses. In obstructive lung diseases this imbalance is potentially associated with disease development and severity. It may also contribute to acute worsening of these diseases, called exacerbations, although there is considerably less data available. In this review we present the current state of knowledge on the contribution of oxidative stress to exacerbations, with a focus on lung macrophages.

Obstructive lung diseases and macrophages

Lung macrophages have been shown to be involved in the induction and progression of lung inflammation in asthma and COPD, but are also emerging as important cells that control and limit inflammatory events in the lung (24, 73, 151, 161). This multitude of different, and sometimes even opposing, tasks is handled through distinct polarized “activation” states of macrophages. Signals from the tissue surrounding macrophages determine the polarization type and prepare them for the different roles needed at specific times.

In the past macrophage polarization was seen as a dichotomous process yielding either M1 and M2 macrophages, similar to the process of differentiation seen for T cells. M1 macrophages or classically activated macrophages are pro-inflammatory macrophages associated with Th1 inflammation. M2 or alternatively activated macrophages are associated with Th2 inflammation and wound healing. However, we now know that this process of polarization is much more

complex *in vivo* and an almost continuous spectrum of different macrophage phenotypes exists. This has made literature from this field rather confusing and in 2014 a consortium of macrophage experts suggested a new nomenclature in which macrophages in *in vivo* situation should be labeled with the markers used to isolate/characterize them (127). Since this usually involves many markers, readability remains an issue and often people still refer to the old M1/M2 names. While writing this review we struggled with old papers using the old names, new papers ignoring the guidelines, papers using the nomenclature correctly and how to summarize results from papers using different markers that can identify macrophages with roughly similar functionalities. We therefore chose to divide lung macrophages first into alveolar macrophages (AMs) when this specific type was mentioned or lung macrophages when no distinction was made. We did not find publications specifically looking at interstitial macrophages (IMs) in the context of oxidative stress and asthma or COPD. Regarding polarization, we grouped macrophages in studies stating the use of M1 or markers associated with Th1 responses under the name M1 and macrophages in studies stating the use of M2 or markers associated with Th2 inflammatory responses under the name M2. As the name "M2" macrophages in literature is also used for macrophages with anti-inflammatory functions we also introduced a third class named M2-like anti-inflammatory macrophages to indicate macrophages that look like M2 macrophages but produce anti-inflammatory or pro-resolution molecules and used this name whenever it was clear that anti-inflammatory macrophages were studied. The different markers used in literature to identify differentially polarized macrophages in human and murine lung tissue are summarized in **Figure 1**. To assist the reader further, we summarized all papers that cite macrophage polarization in **Table 1** and indicated which markers were used for identification and which names these macrophages were given in the original paper.

The role of macrophage polarization in respiratory diseases has been extensively reviewed by us before (22). In short, both asthma and COPD are characterized by alterations in macrophage polarization, and therefore function, that contribute to development and severity of the disease

(23, 51, 54-56, 81, 122, 146). Lung macrophages in healthy individuals or mice have low expression of markers indicating a specific polarization type and most are characterized as anti-inflammatory expressing interleukin (IL)-10 (54, 122). In asthma, however, the numbers of M1 and M2-polarized macrophages are higher than in controls at the apparent cost of M2-like anti-inflammatory macrophages that are lower in asthma compared to control (54, 55, 72, 102, 119, 121, 122, 125). When these IL-10-producing M2-like macrophages are subsequently reinstated in murine lung tissue, this was associated with having less allergic lung inflammation (53). Furthermore, neutrophil-dominated asthma is associated with M1-polarized macrophages, whereas eosinophil-dominated asthma is associated with M2-polarized macrophages in mice (54, 56, 122, 146). These studies combined suggest that in mouse models of asthma lung macrophages lose their anti-inflammatory properties and acquire a polarized activation state with the polarization type determining the inflammation outcome: M1-polarized being associated with neutrophils and M2-polarized with eosinophils. However, this still needs to be confirmed in humans.

In COPD, polarization changes are less apparent, though dysregulation of M1 and M2 polarization patterns has been described with macrophages acquiring and losing both M1 and M2 markers and an unexpected loss of inflammatory signatures in AMs of COPD patients compared to non-COPD smokers (9, 156, 187). A study by Eapen et al. characterized both AMs and IMs from COPD patients, smokers with normal lung function and healthy controls and found that smokers primarily had M1-polarized IMs and M2-polarized AMs compared to nonsmokers irrespective of having COPD (61). The effects of smoking in this study thus appeared to have far more influence on macrophage polarization than having COPD, suggesting that maybe we need more functional readouts to capture the changes in COPD. Indeed, several studies showed changes in AM function as compared to controls (23, 79, 81). For instance, macrophage responsiveness in COPD seems to be impaired, resulting in disturbed efferocytosis of airway epithelial cells and eosinophils (63, 80). In addition, impaired phagocytosis of pathogens by

(alveolar) macrophages was demonstrated in COPD patients (12-15, 17, 165, 185). Summarizing these results, COPD appears to be characterized by dysfunctional macrophages with maybe an inability to polarize effectively towards a specific inflammatory signature, resulting in defective phagocytosis and efferocytosis. This may then contribute to ongoing inflammation due to persistence of dead cells and microbes.

Obstructive lung diseases and oxidative/nitrosative stress

Also characteristic for both asthma and COPD is the presence of oxidative stress. Lung tissue is continuously exposed to ambient air and due to its large surface area and blood supply highly susceptible to oxidative injury by reactive species, including superoxide, hydrogen peroxide (H_2O_2), nitric oxide (NO) and peroxynitrite. These oxidants and nitrating agents can be of either exogenous (e.g. cigarette smoke and air pollution) or endogenous origin (e.g. production by resident and inflammatory cells such as macrophages and in mitochondria). In normal conditions, ROS/RNS act as signaling molecules to regulate physiological processes. Yet, in the case of chronic inflammation, the excess generation of reactive species can also lead to oxidative stress, damaging multiple cellular organelles and processes and ultimately contributing to the pathogenesis and exacerbation of obstructive lung diseases (**Figure 2**, upper panel).

In order to have such an impact, ROS/RNS must outcompete a wide range of antioxidant defense mechanisms, including the glutathione (GSH) and thioredoxin (TRX) redox systems, catalase (CAT) and superoxide dismutase (SOD) enzymes (142). These antioxidant defenses are regulated by nuclear factor erythroid 2-related factor 2 (Nrf2), the master regulator of antioxidant responses (**Figure 2**, lower panel) (195).

Direct measurement of ROS/RNS is relatively complicated because of their high reactivity and short lifetime. As a result, lipid peroxidation products (e.g. 4-hydroxynonenal (4-HNE), 8-isoprostane and/or F_2-isoprostanes and malondialdehyde (MDA)), products of protein oxidation/nitration (e.g. protein carbonylation (this includes e.g. 4-HNE and MDA protein

adducts, resulting from a phenomenon often referred to as carbonyl stress), bromotyrosine, chlorotyrosine and nitrotyrosine) and products of DNA oxidation (e.g. 8-hydroxy-2'-deoxyguanosine (8-OHdG)) have been widely used as (indirect) markers of oxidative and nitrosative damage and thus ROS/RNS activity. Still, one has to keep in mind that proper storage and prevention of further oxidation are important to obtain reliable results.

The role of oxidative stress in the pathogenesis of asthma and COPD has been extensively addressed in several reviews (42, 95, 120, 140, 149). In short, it has been found that excess production of ROS can contribute to airway inflammation and hyperresponsiveness and may also be involved in decreasing sensitivity to treatment and subsequently worsen disease outcomes. Higher levels of markers of oxidative stress have been found in asthmatics and COPD patients versus healthy controls and altered levels of various antioxidants have been reported in asthma and COPD as well (128, 129). An increase in antioxidant capacity is generally explained as an attempt to a defense response, while a decrease most likely represents neutralization or inactivation by ROS. Loss of antioxidants can thus be the consequence of enhanced oxidative stress, but can in turn also contribute to more oxidative stress and perhaps the severity of asthma and COPD. This apparent contradiction in outcomes can only be solved by studying fluctuations in oxidative stress over time and relate these to clinical symptoms in patients.

Nitrosative stress in asthma and COPD is less often investigated. A few studies have looked into the end products of nitrosative stress and found NO concentrations and the severity of eosinophilic airway inflammation to be positively correlated in asthma and a subgroup of COPD patients (52, 199). In addition, exhaled breath condensate (EBC) and sputum peroxynitrite levels were found to be higher and peroxynitrite inhibitory activity lower in asthma and COPD patients compared to healthy volunteers and peroxidative stress was negatively correlated with the forced expiratory volume in one second (FEV_1) (11, 89, 90, 136). This suggests that RNS may have a functional role in asthma and COPD as well. Other evidence suggests that a reduced

availability of arginine may result in higher nitrosative stress with a possible negative impact on lung function in asthma and COPD (38, 148, 152, 153).

Oxidative/nitrosative stress and macrophages in asthma and COPD

Oxidative and nitrosative stress and macrophages are linked in many ways in asthma and COPD. ROS/RNS can affect macrophage function and thereby influence disease severity, but on the other hand the high number of (activated) AMs present in these diseases can contribute to generation of ROS/RNS during phagocytosis or after stimulation with a wide variety of (microbial) agents (a process referred to as the respiratory burst) (69). One of the proteins shown to play a role in bacterial killing by generating ROS in macrophages is tartrate resistant acid phosphatase (145). We have recently shown that the expression of tartrate resistant acid phosphatase is higher in AMs of asthma and COPD patients than in controls, thereby possibly contributing to generation of oxidative stress (23). This is corroborated by the finding that macrophages of patients with asthma and COPD have higher production of inducible NO synthase (iNOS) than nonsmoking and smoking control subjects, resulting in upregulation of RNS as assessed by nitrotyrosine, iNOS and heme oxygenase 1 (HO-1) staining in lung tissue (2, 90, 115, 160, 178).

Other studies have shown that exposure to excess ROS/RNS can lead to impaired function of macrophages, e.g. senescence and impaired phagocytosis (8, 77, 198). This macrophage dysfunction was suggested to at least partially result from oxidation of mannose binding lectin, a key component required for effective phagocytosis (168). Oxidative stress may additionally cause accumulation of damaged lipid proteins in mouse models of COPD, which can inhibit the phagocytic function of AMs and drive inflammatory behavior (126, 166, 167). High oxidative stress in animal models was indeed shown to attenuate AM function, primarily resulting in reduced phagocytic capacity and cell viability (30, 31, 33). Moreover, high oxidative stress affected maturation of AMs in guinea pigs, as demonstrated by a shift towards a less terminally differentiated population (33). Increased ROS production in the AM cell line NR8383 also

resulted in enhanced expression of M2 activation markers, possibly due to induction of transforming growth factor beta (TGF-β) signaling and diminished antioxidant availability (32). Treatment with antioxidants in this case was able to lower oxidative stress and improve phagocytosis and maturation of AMs and partially blocked alternative activation in NR8383 cells (31-33). Further research into specific mechanisms causing impaired AM function showed a key role for NADPH oxidases and mitochondrial ROS (mROS) generation, which in addition provided targets for normalizing ROS production and rescuing phagocytic capacity (110, 111, 190, 191). Although the aforementioned animal studies demonstrate that high oxidative stress plays a role in AM dysfunction, all models are based on chronic alcohol ingestion and more direct evidence is essential to fully understand what happens in asthma and COPD. It was already shown that AMs from COPD patients have chronic mROS production, causing increased mROS baseline levels. However, these AMs fail to generate sufficient mROS upon bacterial challenge (17). High oxidative stress in COPD may thus impair mitochondrial function and result in reduced bacterial clearance. Furthermore, the mitochondrial-specific antioxidant mitoTEMPO did not increase intracellular bacterial numbers in AMs from COPD patients (while it did in healthy), confirming mitochondrial dysfunction as a key determinant of their defective antimicrobicidal response (17).

In addition to endogenous ROS/RNS, the function of macrophages can be altered by exogenously generated ROS/RNS. Cigarette smoke models are commonly used for studying AMs in COPD with cigarette smoke inducing oxidative stress. Cigarette smoke exposure *ex vivo* resulted in a redox imbalance with higher production of NO by rat AMs and higher ROS production by human and mouse macrophages (96, 139, 192). Similar results were found *in vivo* when oxidative stress was assessed as increased expression of MDA and HO-1 and by decreased GSH levels in macrophages of cigarette smoke-exposed rats (183). Moreover, cigarette smoke provokes oxidative damage in macrophages. For example, cigarette smoke exposure resulted in cell apoptosis and downregulated phagocytic ability of macrophages and decreased efferocytosis as measured in both bronchoalveolar lavage fluid (BALF) and tissue macrophages obtained from cigarette

smoke-exposed mice (81, 139, 192). These cigarette smoke-induced changes were shown to improve by procysteine antioxidant treatment (81).

Taken together, these studies suggest that in addition to being an important source of ROS/RNS, the redox state is crucial for proper macrophage function as well as differentiation when needed. The airway inflammation and altered function and polarization of macrophages as seen in asthma and COPD thus may be related to increased oxidative stress found in these diseases. However, it is still not clear whether changes in macrophage polarization are cause or effect of oxidative stress and what the actual consequences are.

Exacerbations of asthma and COPD

Both asthma and COPD patients can suffer from periodic acute worsening of symptoms called exacerbations, that are associated with increased airway inflammation, a decline in lung function and increased mortality. Despite more therapeutic intervention and medication, these remain difficult to control (6, 40). During an exacerbation, patients have difficulties in breathing, chest pain and cough up sputum, caused by restriction of the airways and overproduction of mucus (182). Exacerbations are predominantly triggered by viral and bacterial respiratory infections, but can also be induced by exposure to allergens, air pollution or exercise (101). Yet, why some patients develop an exacerbation during an infection or other exposures and why some do not, is not understood. It has been suggested this may be associated with different levels of oxidative stress.

Oxidative stress during exacerbations of asthma and COPD has been studied in various settings, in humans as well as in animal models. Numerous studies in patients suffering from acute exacerbations requiring hospitalization demonstrated that exacerbations are associated with an increase in oxidative stress, both locally and systemically, as assessed as increases in the levels of well-known oxidative stress markers (i.e. 8-isoprostane, H_2O_2, MDA, protein carbonylation

and reactive oxygen metabolites (ROM)) compared to stable disease (**Table 2**). These increases are often accompanied with higher levels of inflammatory markers such as C-reactive protein (CRP), cysteinyl leukotrienes (Cys-LTs) and leukotriene B4 (LTB4) (3, 7, 18, 116, 159, 193). Experimental allergen or rhinovirus-induced exacerbations in asthmatics and COPD patients were also shown to result in ROS generation and higher levels of 8-isoprostane and/or F_2-isoprostanes compared to baseline (34, 36, 59, 60, 68). Even in an *ex vivo* lipopolysaccharide (LPS)-induced human COPD exacerbation model, higher H_2O_2 and MDA levels were detected compared to vehicle (39). Moreover, animal models of asthma and COPD exacerbations displayed similar increases in oxidative stress levels as reported for patients, indicating that these models are suited to study mechanistic effects. For example, LPS, diesel exhaust particulates, ozone and graphene oxide were all able to exacerbate airway inflammation in ovalbumin or house dust mite mouse models of asthma (both acute and chronic models), resulting in increased ROS production and elevated levels of e.g. 8-isoprostane and MDA (58, 85, 94, 99, 134, 154). In addition, viral infection mimicked by poly(I:C) stimulation led to enhanced protein carbonylation in a mouse model of COPD exacerbation (164).

The majority of human studies on this topic have focused on oxidative stress markers in serum, plasma or material derived from upper or lower airways. Wu et al., however, found that changes in oxidative stress during exacerbations in asthmatic adults can also be detected by measuring the major urinary metabolite of F_2-isoprostane (186). Still, some matrices may have superior clinical utility over others, since discrepancies are known to exist as well. For example, sputum MDA levels in COPD patients experiencing an acute exacerbation were significantly higher compared to stable COPD, healthy controls and after treatment, while levels of MDA in EBC were comparable for all groups (4). The authors hypothesized that this difference may be explained by the high day-to-day variability in EBC MDA readings. On the other hand, a significant association between local and systemic MDA was found in patients experiencing acute COPD exacerbations (194).

Although most studies investigate markers of oxidative stress, antioxidant responses have been studied as well. Significant negative relationships between MDA levels and GSH, glutathione peroxidase (GPx) and SOD were observed in both asthma and COPD exacerbations, implicating an important role for antioxidants in the development of exacerbations (45, 194). **Table 3** depicts some of the most common antioxidants measured in patients hospitalized due to asthma and COPD exacerbations. While it is obvious that levels of markers of oxidative stress are higher during acute exacerbations (**Table 2**), findings regarding antioxidant capacity appear to be conflicting, with some studies finding higher and some finding lower levels than in stable disease. These different outcomes are difficult to explain and can probably only be resolved by following patients clinically in detail over time. Results from experimental and *ex vivo* human exacerbation models were more unanimous, revealing a decrease in GSH and SOD during experimental exacerbations compared to baseline (39, 43, 59). Lower antioxidant levels of CAT, GSH and SOD were also found during exacerbations in mouse models (58, 99, 154). The importance of antioxidant status is further highlighted by *ex vivo* and animal studies showing that the administration of antioxidants (apocynin, curcumin, ebselen, GSH, N-acetylcysteine (NAC) and vitamin E) is to various degrees able to restore antioxidant levels, lower oxidative stress and thereby reduce airway inflammation and hyperresponsiveness and ameliorate the induced exacerbation (39, 58, 62, 99, 135, 154).

Loss of lung function is an important indicator of a developing exacerbation and changes in FEV_1 in relation to oxidative stress and antioxidant levels have therefore been studied as well. Markers of oxidative stress in serum (MDA and ROM) were found to negatively correlate with FEV_1 during asthma and COPD exacerbations (26, 132). Moreover, sputum MDA levels primarily decreased in those COPD patients who had a more pronounced improvement in FEV_1 post-treatment, while MDA levels remained high in patients with minor changes in FEV_1 (4). This suggests that high oxidative stress levels are linked to more severe exacerbations and that the

capacity to counter ROS production is linked to a response to treatment. In addition, it has been suggested that antioxidant levels may reflect the severity of an exacerbation. A significant positive association between SOD activity and FEV_1 was seen in asthma patients admitted to the hospital because of acute exacerbations, suggesting that patients with higher SOD levels are better off during an exacerbation (91). On the other hand, serum levels of TRX negatively correlated with FEV_1 during exacerbations (189). Thus, altered antioxidants during asthma and COPD exacerbations may be part of the pathophysiological features of the disease.

Nitrosative stress during exacerbations remains poorly investigated, although elevated levels of nitrotyrosine were reported during both asthma and COPD exacerbations (68, 85, 171). In addition, acute exacerbations of COPD are characterized by higher levels of NO inhibitor asymmetric dimethylarginine (ADMA) concentrations in serum (148). ADMA promotes the formation of peroxynitrite and results in a shift towards L-arginine breakdown, contributing to airway obstruction. High ADMA levels in these patients were also found to be associated with higher all-cause mortality (180).

Macrophages may contribute to the development of exacerbations in several ways (**Figure 3**). Their defective phagocytic capacity as seen in asthma and COPD can result in impaired clearance of bacteria, subsequently leading to an increased bacterial burden in the lung (12, 67, 76, 112). Defective opsonic phagocytosis by AMs has recently been associated with both exacerbation frequency and FEV_1 in COPD patients (16). Impaired antiviral responses have been seen in asthmatic patients as well, which may be caused by changes in macrophage polarization. M1 macrophages are favorable during viral infections as they have better antigen-presenting and antiviral capacity, but many macrophages in asthma display signs of M2 polarization (118, 122). Several studies have indeed demonstrated that rhinovirus-induced antiviral type 1 responses by AMs are defective in asthma patients (44, 105, 163). In addition to stimulating less M1 polarization, this virus was also demonstrated to exacerbate Th2-mediated airway inflammation

in asthma, which correlated with viral load and symptom severity (86, 123). Moreover, rhinovirus infection in ovalbumin-sensitized mice resulted in more M2 macrophage polarization, enhancing hyperresponsiveness (82). In AMs of COPD patients, M1-related inflammatory genes are downregulated and M2-associated genes are upregulated compared to healthy controls, suggesting a similar effect on the antiviral capacity as seen in asthma (156). Moreover, impaired AM efferocytosis contributes to the accumulation of apoptotic material that may perpetuate inflammation in the airways (158, 168, 179). Impaired efferocytosis of eosinophils in COPD patients was in fact related to both the frequency and severity of future exacerbations (63). In addition, AMs of COPD patients prone to exacerbations were demonstrated to have impaired innate immune responses towards respiratory pathogens, including diminished cytokine induction and reduced nuclear factor kappa B (NF-κB) translocation (13).

Besides macrophage involvement in the induction of exacerbations, emerging evidence points towards changes in function and polarization of macrophages during exacerbations as well, which could be the result of being in an environment of high oxidative stress. Allergen provocation in atopic asthma patients induced airway inflammation and was associated with an altered phenotype pattern within the AM population (107, 108). For example, AMs post-challenge showed increased expression of the cluster of differentiation (CD) molecules CD11b and CD14, potentially resulting from an influx of blood monocytes. In ovalbumin and rhinovirus-induced acute exacerbation mouse models of chronic asthma, macrophage polarization was skewed towards M2/alternative activation, accompanied by higher expression of cell surface markers related to antigen presentation than in control asthmatic mice (35, 41, 131). Moreover, macrophages in mouse models of acute exacerbations exhibited higher expression of several pro-inflammatory cytokines compared to chronically challenged animals (35, 78, 133, 150). Consequently, these AMs were demonstrated to have a greater ability to stimulate the expression of Th2 cytokines when co-cultured with pulmonary $CD4^+$ T lymphocytes (78). In addition, THP-1-derived macrophages displayed an M2-polarized phenotype upon incubation

with sputum from exacerbating COPD patients (75). The altered macrophage function and polarization towards M2 during exacerbations may thus influence immune responses and contribute to aggravation of airway inflammation. This together with the aberrant M1 macrophage differentiation may impair antiviral responses, making it an interesting therapeutic possibility to prevent virus-induced exacerbations.

What causes oxidative/nitrosative stress in exacerbations?

Several factors may contribute to oxidative stress during asthma and COPD exacerbations (**Figure 4**). As mentioned previously, exacerbations are usually caused by exogenous stimuli. Some of these triggers, including cigarette smoke and air pollution, contain different populations of free radicals and ROS/RNS that not only directly contribute to oxidative stress generation in the lung, but also stimulate the production of reactive species by e.g. epithelial cells and phagocytes. More specifically, it has been suggested that various sources of pollution particles trigger oxidant responses in a cell-specific manner (10). Furthermore, pollens were demonstrated to have intrinsic NADPH oxidases and are therefore able to generate ROS (5, 21). Environmental factors thus exacerbate airway inflammation and increase cellular ROS levels, but have been demonstrated to induce oxidative damage to mitochondria as well (66, 109). The resulting mitochondrial dysfunction and enhanced mROS generation was suggested to be responsible for the exacerbation of allergic airway inflammation in mice, as evidenced by the accumulation of eosinophils, mucus hypersecretion and bronchial hyperresponsiveness (1). Thus, exogenous events may directly and indirectly influence oxidative stress levels, thereby contributing to the development of asthma and COPD exacerbations.

Inflammatory cells represent an important endogenous source of ROS. Both asthma and COPD exacerbations are characterized by eosinophil and/or neutrophil recruitment to the airways (138). Following allergen-induced exacerbations in allergic asthmatic patients, circulating eosinophils display enhanced ROS production together with diminished apoptosis (65, 104).

Both observations point towards eosinophil priming upon exposure to allergen. *In vitro* allergen challenge of peripheral neutrophils obtained from allergic asthmatics induced the release of myeloperoxidase (MPO) and ROS production in an allergen-specific, dose and time-dependent manner (70, 124). Likewise, blood and sputum neutrophils of exacerbating COPD patients showed increased ROS production (176).

In addition to neutrophils and eosinophils, AMs are also relevant ROS-producing effector cells that are present in lung tissue during asthma and COPD exacerbations. AMs of allergic subjects and mild asthmatics demonstrated higher ROS metabolism and superoxide production after allergen challenge (36, 37). This may be related to lower Nrf2 activity, because inducing an experimental exacerbation by segmental allergen challenge in human atopic asthmatics led to lower Nrf2 DNA-binding activity and protein expression as well as inhibition of the Nrf2-dependent gene SOD-1 in AMs as compared to baseline (59). Likewise, oxidative stress was higher and protein levels of Nrf2 and its downstream target HO-1 were lower in ozone-exacerbated asthmatic mice than in mice with ovalbumin-induced asthma only (58). Human AMs after allergen challenge were also unable to respond to Nrf2-inducing agents like 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) and sulforaphane *ex vivo*, as exemplified by failure to induce DNA-binding activity or protein expression of Nrf2 (59). This loss of Nrf2 activity and protein seems to be mediated by ROS, since vitamin E supplementation not only resulted in lower oxidative stress but was also able to restore the drop in Nrf2 (58, 59). Moreover, Nrf2 agonists were able to increase phagocytosis by AMs from COPD patients, a process that is defective and associated with impaired responses to oxidative stress in this disease (16). Cigarette smoke-exposed Nrf2-deficient mice demonstrated lower pathogen clearance by macrophages, enhanced airway inflammation and greater pulmonary injury upon bacterial and viral infections than air-exposed mice, emphasizing the importance of Nrf2 in combating oxidative stress (76, 188). Additionally, virus infection in mice attenuated expression of Nrf2 and its target genes, leading to oxidative damage in the lung (83). Impaired Nrf2 activity and

subsequent deterioration of essential antioxidant responses in the airways may therefore play a critical role in the molecular pathways of asthma and COPD exacerbations. Targeting the Nrf2 pathway using e.g. sulforaphane has already been suggested as a tool in preventing exacerbations of COPD, though not all trials were proven successful (19, 25, 76, 87, 184, 195).

Clinical relevance and therapeutic strategies

Measuring oxidative stress levels or altering stress levels are being investigated as clinical approaches in trying to predict, prevent and/or diminish the severity of exacerbations. For example, ROM levels in serum from asthmatics being more likely to experience severe exacerbations were higher compared to patients who did not suffer from exacerbations (132). This finding was supported by a ROC analysis that demonstrated an association between ROM levels and the occurrence of severe exacerbations. ROM levels were also found to be predictive for exacerbations in COPD patients with repeating exacerbations, since they increased before the exacerbation and changed corresponding to clinical symptoms (97). Other oxidative stress markers like lipid peroxide (LPO), MDA-modified low-density lipoprotein (MDA-LDL) and urinary 8-OHdG displayed trends similar to ROM, although changes in MDA-LDL levels appear 3-5 days later, limiting its use as a predictive marker. The activity of SOD has not been found to follow clinical symptoms and only showed minimal fluctuation (97). EBC 8-isoprostane levels, on the other hand, may have some predictive value as Keskin et al. showed that these were higher in asthmatic children with more than four exacerbations per year than in children with only 1-4 exacerbations per year, suggesting that these values are related to the number of exacerbations per year (92). In addition, specific eosinophil-catalyzed protein oxidation may be of important value, since higher baseline urinary levels of bromotyrosine in children corresponded to a fourfold higher chance of the occurrence of an asthma exacerbation (181).

Several studies have found a significant relationship between vitamin D (a membrane antioxidant) insufficiency and higher odds of severe asthma exacerbations (20, 27-29, 147). This effect was even greater by traffic-related air pollution or co-occurrence of folate deficiency (20,

147). More specifically, vitamin D insufficiency was associated with significantly elevated oxidative stress levels, poorer lung function and decreased responsiveness to corticosteroids during severe exacerbations compared to vitamin D sufficiency (27, 103). However, vitamin D deficiency and exacerbations did not show any correlation in COPD cohort studies and it was also found to not increase the risk of rhinovirus-induced exacerbations (100, 141). The effects of vitamin D may possibly be minor in comparison to other complex factors that influence susceptibility to COPD exacerbations.

Taken together, measuring markers of oxidative stress and/or levels of antioxidants may help in identifying patients at risk of (severe) exacerbations of asthma and COPD. This has previously been suggested for allergen sensitization and also for allergen-induced asthma exacerbations (114, 175, 177). Whether these patients will actually benefit from strategies aiming for reduced oxidative stress levels or an increased antioxidant capacity remains to be investigated. Furthermore, studies on the predictive value of oxidative stress levels remain scarce and are mostly conducted with limited patient numbers and over a short time frame. Further research including larger patient cohorts is thus necessary to validate these findings and identify potential biomarkers for predicting exacerbations.

Antioxidant administration to counteract oxidative stress and thereby possibly prevent asthma and COPD exacerbations or modulate their severity has been investigated in quite a few studies. Animal and *ex vivo* studies showed that administration of antioxidants normalized ROS production and antioxidant responses and incidentally also led to improvements in macrophage function and polarization (31, 33, 39, 58, 62, 76, 84, 99, 135, 154). Several clinical studies have investigated the effect of antioxidant administration on exacerbation rates. In COPD patients, the antioxidant and mucolytic agent carbocysteine was well tolerated and daily administration for one year lowered the number of exacerbations in both placebo-controlled and observational studies (64, 196). The antioxidant activity of erdosteine was already confirmed earlier by lower plasma ROS and 8-isoprostane levels, and it was recently also demonstrated to lower the rate

and duration of COPD exacerbations (46, 47). Long-term high-dose NAC treatment (600 mg twice a day) was safe and able to reduce exacerbation frequency in COPD as well, although this was in particular true for moderate disease severity and high-risk patients (169, 170, 197). However, 600 mg daily NAC was unsuccessful in preventing COPD exacerbations, possibly pointing towards a dose-dependent effect (48). Similar trials in asthma patients are currently lacking and the efficacy of antioxidants in reducing asthma exacerbations therefore remains to be elucidated.

Recent meta-analysis of individual participant data demonstrated that supplemental vitamin D reduced the asthma exacerbation rate and this outcome did not differ across patient subgroups (88). Yet, supplementation was only able to reduce exacerbations in COPD patients with baseline vitamin D concentrations below a certain threshold (93, 106, 117).

Targeting oxidative stress using antioxidants may thus provide a strategy for the reduction and/or prevention of exacerbations, though pre-specified subgroups of patients should probably be considered. Furthermore, evaluating the effects on baseline oxidative stress levels could help understand why not all patients benefit from antioxidant treatment. Evidence regarding the mechanism of action in positive trials of antioxidants is also required to clarify whether it is the antioxidant capacity that is critical in reducing exacerbation rates, since most agents described also have mucolytic and anti-inflammatory properties.

Conclusions

This summary of existing literature shows that asthma and COPD and exacerbations of these diseases are characterized by high oxidative stress and impaired macrophage function. Macrophages have multiples roles in the oxidative stress associated with exacerbations: on the one hand the high numbers of (altered) macrophages in asthma and COPD contribute to generation of ROS/RNS and on the other hand oxidative stress also affects macrophage function and polarization. Oxidative stress is associated with decreased capacity of macrophages to respond to pathogens, caused by decreased phagocytosis and aberrant polarization and this

appears to be crucial in the insufficient initial response to exacerbating stimuli. To date, much of the knowledge on oxidative stress and macrophages has been derived from animal models of exacerbations. Although these may provide mechanistic insights, their actual relevance to human disease is largely unknown. Further study into the interactions between oxidative stress and macrophages in the context of acute exacerbations may give us valuable information on how exacerbations occur and why some obstructive lung patients develop exacerbations while others do not. Ideally, one would map fluctuations in a patient undergoing oxidative stress over time, compare frequent and infrequent exacerbators and find out whether asthma and COPD patients before an exacerbation show evidence of more oxidative stress than before a non-exacerbating respiratory infection or compared to healthy controls experiencing a similar respiratory tract infection. This knowledge may lead to targets, markers and therapeutic strategies to reduce or prevent exacerbations.

Acknowledgements

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516 **Table 1.** Overview of papers that cite macrophage polarization.

Reference	Macrophage	Definition
Human		
Bazzan et al., 2017 (9)	M1	iNOS confirmed by HLA-DR, TNF-α
	M2	CD206, IL-4, IL-13
Draijer et al., 2017 (54)	M1	IRF5
	M2	CD206
	M2-like	IL-10
Eapen et al., 2017 (61)	M1	iNOS
	M2	Arginase, CD163
Girodet et al., 2016 (72)	M0	$CD206^{lo}MHC\text{-}II^{lo}$
	M2	$CD206^{hi}MHC\text{-}II^{hi}$
Gutierrez et al., 2010 (75)	M1	TNF-α, IL-6
	M2	Arginase, CD206
Hodge et al., 2011 (81)	M1	CR-3, CR-4, FcγR1, HLA classes I and II
	M2	Arginase, DC-SIGN
Melgert et al., 2011 (122)	Alternatively activated	CD206, stabilin-1
Mouse		
Bunting et al., 2013 (35)	Alternatively activated	Arginase-1, FIZZ1, CCL24, YM1
Chung et al., 2015 (41)	M2	CD206, CD301, IL-13
Draijer et al., 2013; 2016; 2018 (53, 55, 56)	M1	IRF5
	M2	CD206, YM1
	M2-like	IL-10
Hong et al., 2014 (82)	M1	IFN-γ, TNF-α, IL-12
	M2	Arginase-1, CD206, CD301, YM1, IL-4, IL-13
	M2a	CCL17, CCL24
	M2b	IL-10, CD86
	M2c	CXCL13
Kurowska-Stolarska et al., 2009 (102)	M1	TLR2, IL-12, TNF-α, CXCL10
	Alternatively activated	CD206, YM1, FIZZ1, CCL17, CCL22, CCL24
Moreira et al., 2010 (125)	M2	Arginase-1, FIZZ1, YM1
Nagarkar et al., 2010 (131)	M2/alternatively activated	Arginase-1, FIZZ1, YM1, TNF-α, p70 IL-12, MGL-2, IL-10
Robbe et al., 2015 (146)	M1	IRF5
	M2	YM1
	Anti-inflammatory	IL-10

517 Abbreviations: iNOS = inducible nitric oxide synthase, HLA = human leukocyte antigen, TNF-α = tumor necrosis factor α, CD = cluster of
518 differentiation, IL = interleukin, IRF5 = interferon regulatory factor 5, MHC = major histocompatibility complex, CR = complement receptor,
519 FcγR1 = Fc gamma receptor 1, DC-SIGN = dendritic cell-specific intercellular adhesion molecule grabbing non-integrin, FIZZ1 = found in
520 inflammatory zone 1, CCL = chemokine (C-C motif) ligand, YM1 = chitinase 3-like 3, IFN-γ = interferon γ, CXCL = chemokine (C-X-C motif)
521 ligand, TLR2 = toll like receptor 2, MGL-2 = macrophage galactose N-acetyl-galactosamine specific lectin 2
522

Table 2. Overview of oxidative stress markers during acute exacerbations of asthma and COPD.

Marker	Reference	Material	Observation	P
Asthma				
8-isoprostane	Zanconato et al., 2004 (193)	EBC	↔ (n=9) vs. stable asthma (n=13)	NS
	Baraldi et al., 2003 (7)	EBC	↑ vs. after 5 d prednisone treatment (n=15)	<0.05
	Mak et al., 2013 (116)	Plasma	↑ vs. remission (n=18)	<0.01
MDA	Corradi et al., 2003 (45)	EBC	↑ vs. after 5 d prednisone treatment (n=12)	0.001
	Nadeem et al., 2005 (130)	Plasma	↑ (n=32) vs. stable asthma (n=71)	<0.05
	Rahman et al., 1996 (143)	Plasma	↑ (n=11) vs. stable asthma (n=9)	<0.05
	Gumral et al., 2009 (74)	RBCs	↑ vs. stable periods (n=16)	<0.01
Protein carbonyls	Nadeem et al., 2005 (130)	Plasma	↔ (n=25) vs. stable asthma (n=73)	NS
	Rahman et al., 1996 (143)	Plasma	↔ (n=11) vs. stable asthma (n=9)	NS
ROM	Suzuki et al., 2008 (162)	Serum	↑ vs. convalescence (n=7)	<0.001
	Suzuki et al., 2008 (162)	Serum	↑ (n=42) vs. stable asthma (n=11)	<0.05
COPD				
8-isoprostane	Antczak et al., 2012 (3)	EBC	↑ vs. stable periods (n=16)	<0.001
	Biernacki et al., 2003 (18)	EBC	↑ vs. after 2 w antibiotic treatment (n=21)	<0.0001
	Tufvesson et al., 2013 (172)	Sputum	↔ vs. stable periods (n=25)*	NS
H_2O_2	Antczak et al., 2012 (3)	EBC	↑ vs. stable periods (n=16)	<0.001
	Oudijk et al., 2006 (137)	EBC	↑ vs. after 7 d intravenous corticosteroid treatment (n=10)	<0.0005
	Gerritsen et al., 2005 (71)	EBC	↑ vs. after 7 d prednisolone treatment (n=14)	0.001
	Dekhuijzen et al., 1996 (49)	EBC	↑ (n=19) vs. stable COPD (n=12)	<0.001
MDA	Antus et al., 2014 (4)	EBC	↔ vs. discharge (n=34)	NS
	Antus et al., 2014 (4)	EBC	↔ (n=34) vs. stable COPD (n=21)	NS
	Zeng et al., 2013 (194)	Plasma	↑ (n=43) vs. stable COPD (n=35)	<0.05
	Stanojkovic et al., 2011 (159)	Plasma	↓ vs. discharge (n=74)	N/A
	Rahman et al., 1997 (144)	Plasma	↑ vs. discharge (n=13)	<0.01
	Rahman et al., 1996 (143)	Plasma	↑ (n=11) vs. stable COPD (n=9)	<0.05
	Gumral et al., 2009 (74)	RBCs	↑ vs. stable periods (n=17)	<0.001
	Tug et al., 2004 (173)	Serum	↑ vs. stable periods (n=24)	N/A
	Antus et al., 2014 (4)	Sputum	↑ vs. discharge (n=34)	<0.05
	Antus et al., 2014 (4)	Sputum	↑ (n=34) vs. stable COPD (n=21)	<0.01
	Zeng et al., 2013 (194)	Sputum	↑ (n=43) vs. stable COPD (n=35)	<0.001
Protein carbonyls	Rahman et al., 1996 (143)	Plasma	↔ (n=11) vs. stable asthma (n=9)	NS
ROM	Komatsu et al., 2007 (97)	Blood	↑ (n=8) vs. chronic stable state (n=10) and recovery (n=6)**	<0.01
	Koutsokera et al., 2009 (98)	Serum	↔ vs. follow-up (n=30)	NS

Observations are defined as an increase (↑), decrease (↓) or no change (↔) in quantified concentrations of oxidative stress markers during acute exacerbations compared to either the same group of patients during recovery, or a separate group with stable disease.

Abbreviations: MDA = malondialdehyde, ROM = reactive oxygen metabolites, EBC = exhaled breath condensate, RBCs = red blood cells, d = days, w = weeks, NS = not significant, N/A = not available

*Stable periods are before the onset of exacerbation

**All from the same n=10, chronic stable state is before the onset of exacerbation

Table 3. Overview of antioxidants during acute exacerbations of asthma and COPD.

Marker	Reference	Material	Observation	P
Asthma				
CAT	Gumral et al., 2009 (74)	RBCs	↑ vs. stable periods (n=16)	<0.001
	Nadeem et al., 2005 (130)	RBCs	↔ (n=32) vs. stable asthma (n=89)	NS
GPx	Nadeem et al., 2005 (130)	Plasma	↔ (n=25) vs. stable asthma (n=83)	NS
	Gumral et al., 2009 (74)	RBCs	↓ vs. stable periods (n=16)	<0.01
	Nadeem et al., 2005 (130)	RBCs	↔ (n=28) vs. stable asthma (n=82)	NS
GRd	Gumral et al., 2009 (74)	RBCs	↓ vs. stable periods (n=16)	<0.001
GSH	Nadeem et al., 2005 (130)	Blood	↔ (n=30) vs. stable asthma (n=86)	NS
	Corradi et al., 2003 (45)	EBC	↓ vs. after 5 d prednisone treatment (n=12)	<0.05
	Deveci et al., 2004 (50)	Sputum	↓ (n=10) vs. stable asthma (n=11)	<0.001
Protein sulfhydryls	Nadeem et al., 2005 (130)	Plasma	↓ (n=32) vs. stable asthma (n=90)	<0.01
	Rahman et al., 1996 (143)	Plasma	↔ (n=11) vs. stable asthma (n=9)	NS
SOD	Katsoulis et al., 2010 (91)	RBCs	↓ vs. discharge (n=38)	<0.001
	Gumral et al., 2009 (74)	RBCs	↔ vs. stable periods (n=16)	NS
	Nadeem et al., 2005 (130)	RBCs	↔ (n=32) vs. stable asthma (n=80)	NS
TEAC	Rahman et al., 1996 (143)	Plasma	↓ (n=11) vs. stable asthma (n=9)	N/A
TRX	Yamada et al., 2003 (189)	Serum	↑ vs. stable periods (n=8)	<0.005
	Yamada et al., 2003 (189)	Serum	↑ (n=26) vs. stable asthma (n=30)	<0.01
COPD				
CAT	Gumral et al., 2009 (74)	RBCs	↔ vs. stable periods (n=17)	NS
GPx	Zeng et al., 2013 (194)	Plasma	↓ (n=43) vs. stable COPD (n=35)	<0.05
	Gumral et al., 2009 (74)	RBCs	↓ vs. stable periods (n=17)	<0.01
	Zeng et al., 2013 (194)	Sputum	↓ (n=43) vs. stable COPD (n=35)	<0.001
GRd	Gumral et al., 2009 (74)	RBCs	↓ vs. stable periods (n=17)	<0.05
GSH	Drost et al., 2005 (57)	BALF	↓ (n=12) vs. stable COPD (n=5)	N/A
	Zeng et al., 2013 (194)	Plasma	↓ (n=43) vs. stable COPD (n=35)	<0.05
	Turgut et al., 2014 (174)	Sputum	↔ (n=11) vs. stable COPD (n=10)	NS
	Zeng et al., 2013 (194)	Sputum	↓ (n=43) vs. stable COPD (n=35)	<0.001
Protein sulfhydryls	Rahman et al., 1997 (144)	Plasma	↓ vs. discharge (n=13)	<0.001
	Rahman et al., 1996 (143)	Plasma	↓ (n=11) vs. stable COPD (n=9)	<0.05
SOD	Zeng et al., 2013 (194)	Plasma	↓ (n=43) vs. stable COPD (n=35)	<0.05
	Stanojkovic et al., 2011 (159)	Plasma	↑ vs. discharge (n=74)	N/A
	Gumral et al., 2009 (74)	RBCs	↑ vs. stable periods (n=17)	<0.01
	Zeng et al., 2013 (194)	Sputum	↓ (n=43) vs. stable COPD (n=35)	<0.001
TEAC	Rahman et al., 1997 (144)	Plasma	↓ vs. discharge (n=13)	<0.05
	Rahman et al., 1996 (143)	Plasma	↓ (n=11) vs. stable asthma (n=9)	N/A

Observations are defined as an increase (↑), decrease (↓) or no change (↔) in quantified concentrations of antioxidants during acute exacerbations compared to either the same group of patients during recovery, or a separate group with stable disease.

Abbreviations: CAT = catalase, GPx = glutathione peroxidase, GRd = glutathione reductase, GSH = glutathione, SOD = superoxide dismutase, TEAC = trolox equivalent antioxidant capacity, TRX = thioredoxin, RBCs = red blood cells, EBC = exhaled breath condensate, BALF = bronchoalveolar lavage fluid, d = days, NS = not significant, N/A = not available

Figure legends

Figure 1. Summary of the M1 (blue) and M2 (grey) polarization concept. Shown are different markers and cytokines that have been used in literature to identify differentially polarized macrophages in the human and murine lung.

Figure 2. Highlights of the oxidative stress pathway and its markers/antioxidants (upper panel). Oxidative stress can lead to lipid peroxidation products, oxidized proteins and/or amino acids and oxidative DNA damage. In cases of overwhelming oxidative responses (R·) and therefore cell and tissue damage by reactive species, Nrf2 translocates to the nucleus, where it binds to antioxidant response elements (ARE) and activates genes involved in the cellular antioxidant and anti-inflammatory defense (lower panel). Under normal conditions, Nrf2 is maintained in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1), resulting in its rapid ubiquitination (ub) and subsequent proteasomal degradation.

Figure 3. Macrophages in the development of asthma and COPD exacerbations. The altered polarization and defective phagocytosis and efferocytosis of macrophages as seen in asthma and COPD results in impaired responses towards exogenous (oxidative) triggers, leading to exaggerated airway inflammation and oxidative stress. Concomitantly, high oxidative stress facilitates an increase in NADPH oxidases, mitochondrial dysfunction and reduced Nrf2 activity, thereby influencing immune responses and contributing to aggravation of inflammation in the airways, further enhanced oxidative stress and exacerbations.

Figure 4. Contributing factors to oxidative stress during exacerbations of asthma and COPD. Environmental stimuli that trigger exacerbations (e.g. air pollution, respiratory pathogens, cigarette smoke and allergens) account for an increase in exogenous ROS. Subsequently, this provokes (mitochondrial) ROS generation by resident and inflammatory cells in the airways and the circulation. Together with the enhanced recruitment of ROS-producing inflammatory cells to

565 the airways, this ultimately leads to the increased oxidative stress and altered antioxidant
566 availability observed during exacerbations. Presented cells are eosinophils (red), neutrophils
567 (purple), monocytes/macrophages (blue) and epithelial cells (green).
568

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